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REMARKS

The last Office Action has been carefully considered.

Regarding Priority

In the last Office Action, the Examiner reasoned that the effective filing date of the claimed invention is 2-11-2000, the filing date of PCT/KR00/00104, because 1999-4860 filed in Korea on 2-11-1999 did not teach isolating EG cells as claimed.

However, referring to the translation of the priority document, the effective filing date should be determined to be 2-11-1999 for the following reasons:

As found in the translation, since the claimed invention is described in the specification and claims of the priority document, the priority should be acknowledged. More specifically, the claimed invention is supported by Examples 1-2 and claims of the priority document and furthermore, the priority document meets the requirements of 35 U.S.C. 112.

Even though claim 1 and Example 1 of the priority document indicate that the PGC colonies are prepared in the first step (i), the product from the first step inherently comprises the EG cells.

Importantly, the steps comprised in the process of the priority document are completely identical to those of the present application, except that the product from the first step (i) has been recognized as PGC colonies in the priority document, while recognized as EG cell colonies in the present application. Such recognition to the intermediate produced in the process for preparing an EG cell line has not been made by characterizing the intermediate. It could be appreciated that the steps (i) and (ii) of the priority document, and the steps (a) and (b) of the present invention are intended steps. Accordingly, such difference in indicating the intermediate produced in a process does not render the inventions described in the priority document and the present application to be different from each other.

Therefore, the present invention is considered to be identical to that described in the priority document and furthermore, supported by the disclosures of the priority document. Consequently, the effective filing date of the claimed invention is 2-11-1999.

Rejection under 35 U.S.C. 112

With respect to the rejections under 35 U.S.C. 112, it is respectfully submitted that the amended claims moot these rejections.

In particular, the Examiner states that the distinction between PGCs and EG cells cannot be determined. However, Example 3 of the present application clearly shows that EG cells established by the present invention possess characteristics of a pluripotent cell. It is well known to one skilled in the art that PGCs are not pluripotent. Therefore, EG cells of the present invention are distinguished from PGCs.

With regard to the term, "differentiation inhibitory factor", its metes and bounds can be found in the present specification (page 6, lines 11-16).

Furthermore, it is generally understood by one skilled in the art that the term, "unit" of LIF, is defined by the amount of LIF required to induce differentiation in M1 colonies in 1 ml agar cultures (Appendix I, col. 1, "Performance Characteristics"). 1 unit of LIF is defined by the amount of LIF required to induce differentiation in 1% of M1 colonies in 1 ml agar cultures. It is noteworthy that the well-known definition of enzyme or protein activity is generally not indicated in patent specification (e.g., WO 99/06534 (Ponce de Leon)). Accordingly, it is respectfully requested that the 35 U.S.C. 112, second paragraph rejection of claims 1-3, 7, 9 and 13 be withdrawn.

Rejection under 35 U.S.C. 102

A claim is anticipated only if each and every element as set forth in claim is found either expressly or inherently described, in a single prior art reference. MPEP § 2131.

(a) Regarding Rejection Based on Pain

The Examiner indicates that Pain teaches isolating blastodermal cells from chicken embryos and culturing the cells to prepare chicken embryonic stem cells. Furthermore, the Examiner asserts that the blastodermal cells are inherently primordial germ cells (PGCs).

However, It is generally recognized that the blastodermal cells include numerous cell types. Therefore, it is unreasonable that the blastodermal cells are inherently considered to be PGCs. Moreover, the present invention employs PGCs isolated from avian embryonic gonad not blastoderm. It is notable that PGCs show different characteristics depending on their isolation source, i.e, their location according to embryo development. For example, alkaline phosphatase activity of avian PGCs is observed as early as 2 days of incubation but not after entry of PGCs into genital ridge as described on page 13, lines 5-13 of the present specification. Therefore, the gonadal PGCs used

in this invention are different from PGCs in blastoderm isolated from embryos at stage IX-XI.

The Examiner asserts that the cells obtained by Pain through long-term culture are embryonic germ (EG) cells. However, this assertion appears groundless.

Firstly, Pain expressly describes embryonic stem (ES) cells rather than EG cells. Referring to the general state of the art to which the present invention pertains, the ES cell is generally classified to three groups according to the origination from which it is derived (Appendix II):

- (i) ES cells derived from blastocyst (blastoderm) or morula;

- (ii) EG cells derived from PGCs; and

- (iii) embryonic carcinoma (EC) cells derived from carcinoma cells.

It could be understood by one skilled in the art that Pain discloses **ES cells** derived from blastoderm but the present invention provides **EG cells derived from PGCs**. In short, the approaches of Pain and the present invention are distinctly different from each other in view of the cell type finally obtained.

Secondly, the conclusion that the cells used are EG cells referring to the mere fact that cells are able to provide germline transmission, is unreasonable. It is well known to one skilled in the art that ES cells are also able to provide germline transmission. In addition, Pain describes that ES cells obtained have the capacity of providing germline transmission at a low frequency; in addition, he indicates that such competent cells are derived from 7-day-old culture as described on page 2344, col.2, last 16 line. It is not certain that the cells cultured for 7 days are determined ES cells.

Thirdly, the EG cells of the present invention and ES cells of Pain were characterized to have respective properties. In particular, the EG cells of the present invention were examined to show a positive reaction in Periodic acid-Shiff's staining as indicated in Example 3-1 of the instant application. Such positive reaction strongly demonstrates that the cells obtained from this invention are originated from PGC. Importantly, it can be found that the EG cells of the present invention and ES cells of Pain show other pattern in terms of endogenous alkaline phosphatase activity. The EG cells of the present invention exhibit little or no alkaline phosphatase activity as described in Example 3-4 of the instant specification, demonstrating the

EG cells of the present invention are derived from gonadal PGCs; however, the ES cells of Pain show a strong alkaline phosphatase activity as indicated in page 2341, col.2, lines 6-18.

Lastly, WO99/06534 discloses on page 15, lines 9-13:

Although not wishing to be held to this hypothesis, the cells of the present invention are believed to be EG cells because they are derived from PGC's and not from the blastoderm as are ES cells.

The above disclosure demonstrates that one skilled in the art generally understands that EG cells derived from PGCs are different from ES cells from blastodermal cells.

In view of the foregoing, it could be appreciated that the EG cells of the present invention are distinctly different from the ES cells of Pain.

The present invention uses a mitotically active feeder layer in culturing and subculturing EG cells. Such feature is not found in Pain. Furthermore, Pain employs STO feeder cells treated with mitomycin C, i.e., mitotically inactive feeder cells (page 2340, "Preparation of culture dishes and feeder cells"). It is noteworthy that **the mitotically active feeder layer** is not generally used for culturing stem cells or embryonic cells. It is well known to one skilled in the



art that the culture of stem cells or embryonic cells necessitates **the mitotically inactive feeder layer** (Appendix III, page 290, lines 4-7 and page 291, Fig. 1; Appendix IV, page 155, col. 1, lines 8-11; Appendix V, page 29, lines 17-18; and Appendix VI, page 554, col.2, lines 34-36). Accordingly, the present invention using a mitotically active feeder layer in culturing EG cells is very unique and unanticipated over Pain as well as the general understandings of those skilled in the art.

Consequently, claims 1-15 and 26 of the present application are not anticipated by Pain because Pain does not expressly or inherently describe each and every element of claims 1-15 and 26.

Furthermore, claims 1-15 and 26 of the present application are unobvious over Pain in light of the following:

(a) different type of cell cultured

The present invention uses gonadal PGCs but Pain uses blastodermal cells.

(b) different type of cell obtained finally

The present invention obtains EG cells but Pain obtains ES cells.

(c) germline chimera production

The EG cells prepared by the present invention successfully produce germline chimeras (Tae Sub Park, et al., Molecular Reproduction and Development, 65:389-395, Tables 1-2). Furthermore, the EG cells are established by subculturing for longer period of time. However, Pain is successful in the production of germline chimera, only when 7-day-old culture cells are used. It is unreasonable that 7-day-old culture cells are considered to be ES cells, as described previously.

(d) other feature

The present invention uses a mitotically active feeder layer in culturing and subculturing EG cells. Such a feature is not found in Pain. Accordingly, it is respectfully requested that the 35 U.S.C. 102(b) rejection of claims 1-15 be withdrawn.

(b) Regarding Rejection Based on Alliolli

In contrast to the Examiner's statement in the last Office Action, Alliolli does not disclose the preparation of EG cells but only the culturing of PGCs.

The Examiner asserts that the PGCs of Alliolli were isolated from the germinal ridge of an avian blastoderm and

were pluripotent. However, the PGCs of Alliolli were isolated from gonad of 5-day-old embryo as described on page 31, col.2, "Collection of PGCs". In addition, the germinal ridge is not present in avian blastoderm but in gonad.

Furthermore, it is unreasonable that the PGCs of Alliolli are determined to be pluripotent because Alliolli never disclose the differentiation potential of PGCs cultured.

The Examiner reasoned that the media used by Alliolli for culturing PGCs contains steel factor, LIF and FGF. However, Alliolli virtually does not use such factors. As described on page 34, col.1, "Proliferation in Cell Culture", Alliolli uses DMEM/F12 medium containing penicillin/streptomycin and glutamine with or without FCS. The culture with steel factor, LIF and FGF is presented only as a prophet example.

Furthermore, it is evident that the cells cultured by Alliolli are not EG cells but PGCs. Long-term culture and subculture necessary to obtain the EG cell line are not performed by Alliolli.

The Examiner reasoned that "culturing the EG cells" and "recovering and subculturing the EG cells" are intended steps that may not occur. However, the amended claim 1 clearly recites that the prepared EG cell line exhibits EG

cell characteristics. Therefore, the steps for preparing EG cell line are not considered to be intended steps.

The present invention uses a mitotically active feeder layer in culturing and subculturing EG cells. Such feature is not found in Alliolli.

Consequently, claims 1-6, 8, 10-15 and 26 of the present application are not anticipated by Alliolli because Pain does not expressly or inherently describe each and every element of claims 1-5 and 26.

Furthermore, claims 1-6, 8, 10-15 and 26 of the present application are not suggested by Alliolli in light of the facts described above for the unobviousness over Pain. It is therefore requested that this rejection be withdrawn.

(c) Regarding Rejection Based on Chang (1995, Cell Biol Internatl. Vol. 19, No. 2, pg 143-149)

Contrary to the Examiner's reasoning, Chang does not disclose the preparation of EG cells but only the culturing of PGCs.

The Examiner stated that the PGCs of Chang are isolated from the genital ridge of day 5 chicken embryo. However, Chang describes that PGCs were separated from the embryonic blood on page 144, col.2, 1<sup>st</sup> paragraph, line 9.

In addition, the Examiner reasoned that the PGCs of Chang are isolated from the gonad of an avian blastoderm and are pluripotent. However, the avian blastoderm does not comprise the gonad. Furthermore, since the PGCs of Chang are not examined to have the differentiation potential, it is not acceptable to one skilled in the art that the PGCs of Chang are pluripotent.

The Examiner asserts that the cell culture was maintained for at least 4 days. However, Chang describes that GR stroma cells were seeded in wells of 96-well culture plates and incubated for at least 4 days before culture with 5-day-old PGCs. Therefore, the cells cultured for at least 4 days are not PGCs but GR stroma cells.

The Examiner reasoned that PGCs isolated from stage 13-14 are equivalent to PGCs isolated from stage 14 as claimed. However, the present invention uses PGCs isolated from the gonad of embryo at stage 20-36 as recited in claim 1. In fact, little or no PGCs are isolated from the gonad of embryo at stage 13-14.

The Examiner stated that "culturing the EG cells" and "recovering and subculturing the EG cells" are intended steps that may not occur. However, amended claim 1 clearly recites that the prepared EG cell line exhibits EG cell

characteristics. Therefore, the steps for preparing EG cell line are not considered to be intended steps.

Furthermore, it is evident that the cells cultured by Chang are not EG cells but PGCs. Long-term culture and subculture necessary to obtain the EG cell line are not performed by Chang. Importantly, Chang does not characterize to demonstrate that PGCs cultured are EG cells.

The present invention uses a mitotically active feeder layer in culturing and subculturing EG cells. Such feature is not found in Chang.

Consequently, claims 1-15 and 26 of the present application are not anticipated by Chang because Chang does not expressly or inherently describe each and every element of claims 1-15 and 26.

Furthermore, claims 1-15 and 26 of the present application are unobvious over Chang in light of the facts described above for the unobviousness over Pain. It is therefore respectfully requested that this rejection be withdrawn.

(d) Regarding Rejection Based on Chang (1997, Cell Biol Internatl. Vol. 21, No. 8, pg 495-499)

Chang does not disclose the preparation of EG cells but

application are not anticipated by Chang because Chang does not expressly or inherently describe each and every element of claims 1-15 and 26.

Furthermore, claims 1-15 and 26 of the present application are unobvious over Chang in light of the facts described above for the unobviousness over Pain. Accordingly, it is respectfully requested that this rejection be withdrawn.

(e) Regarding Rejection Based on Petite (USP 6,333,192)

As described previously, the effective filing date of the claimed invention is 2-11-1999, the filing date of Korean Patent Application. Accordingly, the withdrawal of this rejection is respectfully requested.

If not so, this rejection should be withdrawn for the reasons indicated hereunder.

The Examiner understands that PGCs cultured for 5 days by Petite become ES cells. However, it is generally appreciated by one skilled in the art that the 5-day culture is only a primary culture. Unlikely, the present invention provides an established EG cell line resulted from long-term culturing.

Furthermore, even though Petitte discusses that PGCs cultured could develop an ESC phenotype only depending on anti-SSEA-1 staining and cell appearance results, he could not demonstrate that PGCs cultured are pluripotent and capable of providing a chimeric avian. Accordingly, it would be appreciated by one of skill that PGCs cultured by Petitte are not authentic EG cell, *inter alia*, EG cell line.

Petitte employs a preconditioned STO feeder layer as an essential element as recited in claim 1. However, the present invention does not use a preconditioned STO feeder layer but a mitotically active feeder layer. Since the mitotically active feeder layer is generally used for cell culturing, the present process using it is evidently rendered to be novel and unobviousness.

Consequently, claims 1-6, 8, 10-15 and 26 of the present application are not anticipated by Petitte because Petitte does not expressly or inherently describe each and every element of claims 1-6, 8, 10-15 and 26.

Furthermore, claims 1-15 and 26 of the present application are unobvious over Chang in light of the facts described above for the unobviousness over Pain.



(f) Regarding Rejection Based on Petite (USP 5,340,740), Petite (USP 5,656,479) or Petite (USP 5,840,510)

The Examiner indicates that Petite teaches culturing all the cells from a stage X-XIV embryo and isolating PGCs. However, cells of Petite are distinctly different from PGCs of the present invention. The cells obtained from a stage X-XIV embryo, i.e., blastoderm, comprise numerous cell types. Furthermore, Petite never disclose the isolation of PGCs. Therefore, it is unreasonable that the cells used by Petite are considered to be PGCs. Moreover, the present invention employs PGCs isolated from avian embryonic gonad not blastoderm.

The Examiner describes that Petite seeded the cells onto chicken embryonic fibroblast feeder layers and cultured with BRL conditioned medium.

However, it is notable that Petite discloses on col.7, lines 7-14 of '740; col.6, line 44 of '479; and col.6, line 54-65 of '510:

10 whole embryos at stage X were isolated, dissociated, seeded onto chicken embryonic fibroblast feeder layers, and cultured with 80% BRL-CM. A significant amount of differentiation occurred, mainly cells of a fibroblast-like phenotype. Only a few clusters of

cells remained relatively undifferentiated and contained large amounts of lipid. These cells grew slowly, if at all, and were lost by the second passage.

That is to say, Petite explicitly indicates that the cells from stage X embryo cannot be cultured with chicken embryonic fibroblast feeder layers for obtaining undifferentiated avian cells. For that reason, Petite only claims a method using a mouse fibroblast feeder layer (STO feeder layer).

The present invention successfully cultures PGCs isolated from embryonic gonad to obtain EG cell line not using a mouse fibroblast feeder layer. In this regard, the description of Petite is entitled to be negative teaching. A negative teaching can provide a good evidence of non-obviousness. *Gillette Co. v. S.C. Johnson & Son Inc.*, 919 F. 2d 720, 16 U.S.P.Q. 2d 1923 (Fed. Cir. 1990). The description of Petite teaches away from the use of a feeder layer other than a mouse fibroblast feeder layer for culturing PGCs.

In addition to this, Petite discloses on col.6, lines 6-10:

STO feeder cells are prepared by culturing STO cells to 80% confluency in DMEM with 10% FBS. The cells are then treated with mitomycin C.

only the culturing of PGCs, contrary to the Examiner's indication.

The Examiner's assertion, based on the mere fact that cells are able to provide germline transmission, that the gPGCs of Chang are EG cells is unreasonable. It is well known to one skilled in the art that PGCs injected to recipient are also able to provide germline transmission. Furthermore, it is kindly advised that this article was submitted by the present inventors including TAE SUB PARK and JAE YONG HAN. The present inventors confirm that the gPGCs of Chang are not EG cells.

Moreover, it is evident that the PGCs of Chang are not EG cells but PGCs, in view of the fact that long-term culture and subculture necessary to obtain the EG cell line are not performed by Chang. Chang cultured PGCs only for 5 days, which is considered to be primary culture, as described on page 496, 1<sup>st</sup> paragraph in "RESULTS". Notably, Chang does not characterize to demonstrate that PGCs cultured are EG cells.

The present invention uses a mitotically active feeder layer in culturing and subculturing EG cells. Such feature is not found in Chang.

Consequently, claims 1-15 and 26 of the present

In other words, Petite use mitotically inactive feeder layer obtained by the treatment of mitomycin C. However, the present invention employs a mitotically active feeder layer for culturing EG cells.

The Examiner indicates that "culturing the EG cells" and "recovering and subculturing the EG cells" are intended steps that may not occur. However, the amended claim 1 clearly recites that the prepared EG cell line exhibits EG cell characteristics. Therefore, the steps for preparing EG cell line are not considered to be intended steps. Moreover, the cells cultured by Petite are not PGCs but blastodermal cells obtained from a stage X-XIV embryo.

Consequently, claims 1, 2, 4-15 and 26 of the present application are not anticipated by Chang because Chang does not expressly or inherently describe each and every element of claims 1, 2, 4-15 and 26.

Furthermore, claims 1, 2, 4-15 and 26 of the present application are unobvious over Petite in light of the facts described above for the unobviousness over Pain.

It is therefore respectfully requested that this rejection be withdrawn.

(g) Regarding Rejection Based on Ponce de Leon (USP 6,156,569)

The Examiner indicates that Ponce de Leon teaches isolating PGCs from cells of stage XIV embryos. More specifically, the PGCs of Ponce de Leon are collected from the dorsal aorta of stage 13-14 embryos (col.7, lines 43-44). However, the present invention employs the PGCs from gonad at stage 24-36. Therefore, the PGCs of Ponce de Leon and the present invention are different from each other.

With respect to the culturing method, Ponce de Leon cultures PGCs according to a drop culture (col.7, lines 49-53) but the present invention cultures PGCs according to a co-culture with a feeder cell layer such as germinal ridge stroma cell and avian embryonic fibroblast.

Moreover, Ponce de Leon does not employ a feeder cell layer for culturing but the present invention essentially uses a feeder cell layer. Ponce de Leon says that none of the cell feeder layers improved the long-term culture conditions of the PGCs (col.10, lines 44-45). In this regard, the description of Ponce de Leon is entitled to be negative teaching. The description of Ponce de Leon teaches away from the use of a feeder layer for culturing PGCs.

The Examiner asserts that the PGCs of Ponce de Leon are

pluripotent. However, Ponce de Leon never demonstrates that the PGCs themselves are pluripotent. The fact that the PGCs of Ponce de Leon generate chimeric chickens, cannot demonstrate the PGCs are pluripotent. It is generally understood by one skilled in the art that to demonstrate the pluripotency of a cell, it must be presented that a cell is capable of differentiating into various cell types. Hence, it could be recognized that Ponce de Leon does not disclose and teach EG cells originated from PGCs.

Consequently, claims 1, 2, 4-15 and 26 of the present application are not anticipated by Ponce de Leon because Ponce de Leon does not expressly or inherently describe each and every element of claims 1, 2, 4-15 and 26.

Furthermore, claims 1, 2, 4-15 and 26 of the present application are unobvious over Ponce de Leon in light of the facts described above for the unobviousness over Pain.

It is therefore respectfully requested that this rejection be withdrawn.

(h) Regarding Rejection Based on Ponce de Leon (WO 99/06534)

As described previously, the effective filing date of the claimed invention is 2-11-1999, the filing date of

Korean Patent Application. Accordingly, the withdrawal of this rejection is respectfully requested.

If not so, this rejection should be withdrawn for the reasons indicated hereunder.

The Examiner indicates that Ponce de Leon teaches isolating PGCs from cells of stage XIV embryos. More specifically, the PGCs of Ponce de Leon are collected from the dorsal aorta of stage 13-14 embryos (page 27, lines 25-28). However, the present invention employs the PGCs from gonad at stage 24-36. Therefore, the PGCs of Ponce de Leon and the present invention are different from each other.

With respect to the culturing method, Ponce de Leon cultures PGCs according to a drop culture (page 28, lines 4-8) but the present invention cultures PGCs according to a co-culture with a feeder cell layer such as germinal ridge stroma cell and avian embryonic fibroblast.

Moreover, Ponce de Leon does not employ a feeder cell layer for culturing but the present invention essentially uses a feeder cell layer. Ponce de Leon says that none of the cell feeder layers improved the long-term culture conditions of the PGCs (page 37, lines 13-15). In this regard, the description of Ponce de Leon is entitled to be negative teaching. The description of Ponce de Leon teaches

away from the use of a feeder layer for culturing PGCs.

The Examiner asserts that the PGCs of Ponce de Leon are pluripotent. However, Ponce de Leon never demonstrates that the PGCs themselves are pluripotent. The fact that the PGCs of Ponce de Leon generate chimeric chickens, cannot support the PGCs are pluripotent. It is generally understood by one skilled in the art that to demonstrate the pluripotency of a cell, it must be presented that a cell is capable of differentiating into various cell types. Hence, it could be recognized that Ponce de Leon does not disclose and teach EG cells originated from PGCs.

Consequently, claims 1-13 and 26 of the present application are not anticipated by Ponce de Leon because Ponce de Leon does not expressly or inherently describe each and every element of claims 1-13 and 26.

Furthermore, claims 1-13 and 26 of the present application are unobvious over Ponce de Leon in light of the facts described above for the unobviousness over Pain.

It is therefore respectfully requested that this rejection be withdrawn.



CONCLUSION

In view of the amendments to the claims and the arguments presented herein, it is respectfully requested that each of the rejections in the last Office Action be withdrawn and that the claims remaining in the present application be passed to issue.

Respectfully submitted,

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Audrey de Souza

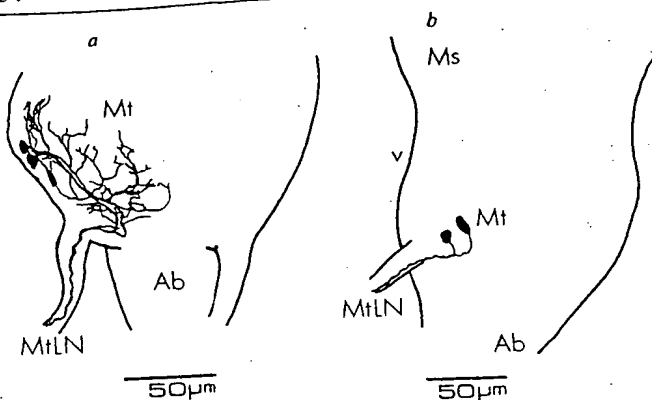


Fig. 3 Two 'intermediate' transformations. *a*, A thoracic ganglion from an animal of genotype *abx bx³ pbx/abx bx³ pbx* in horizontal plane. A metathoracic leg was backfilled and the HRP visualized using method (1) of Fig. 1 legend. *b*, A thoracic ganglion from an animal of the genotype *abx bx³ pbx/Df(3)P2* in sagittal plane, V marking the ventral surface. A metathoracic leg was backfilled and the HRP visualized using method (2) of Fig. 1 legend. Labelling as in Fig. 1.

result from a direct effect of the genes in another tissue, which in turn affects the CNS by induction or by mechanical constraints on CNS growth. Experiments using mosaic flies should determine which tissue must be mutant for the pattern of organization of leg motoneurons to be transformed. This should help to elucidate the mechanisms underlying segmental differences in the CNS.

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1. Lewis, E. B. *Nature* 267, 565-570 (1978).
2. Mesulam, M.-M. *J. Histochem. Cytochem.* 24, 1273-1280 (1978).
3. Hanker, J. S., Yates, P. E., Metz, C. B. & Rustioni, A. *Histochem. J.* 9, 789-792 (1977).
4. Bacon, J. P. & Altman, J. S. *Brain Res.* 138, 359-363 (1977).
5. Power, M. E. *J. comp. Neurol.* 88, 347-409 (1948).
6. Lewis, E. B. *Abstr. 16th Int. Congr. Ent. Kyoto*, 161 (1980).
7. Lewis, E. B. *Drosoph. Inf. Serv.* 55, 208 (1980).
8. Palka, J., Lawrence, P. A. & Hart, H. S. *Dev. Biol.* 69, 549-575 (1979).
9. Ghysen, A. *Nature* 274, 869-872 (1978).

## Establishment in culture of pluripotent cells from mouse embryos

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Pluripotent cells are present in a mouse embryo until at least an early post-implantation stage, as shown by their ability to take part in the formation of chimaeric animals<sup>1</sup> and to form teratocarcinomas<sup>2</sup>. Until now it has not been possible to establish progressively growing cultures of these cells *in vitro*, and cell lines have only been obtained after teratocarcinoma formation *in vivo*. We report here the establishment in tissue culture of pluripotent cell lines which have been isolated directly from *in vitro* cultures of mouse blastocysts. These cells are able to differentiate either *in vitro* or after inoculation into a mouse as a tumour *in vivo*. They have a normal karyotype.

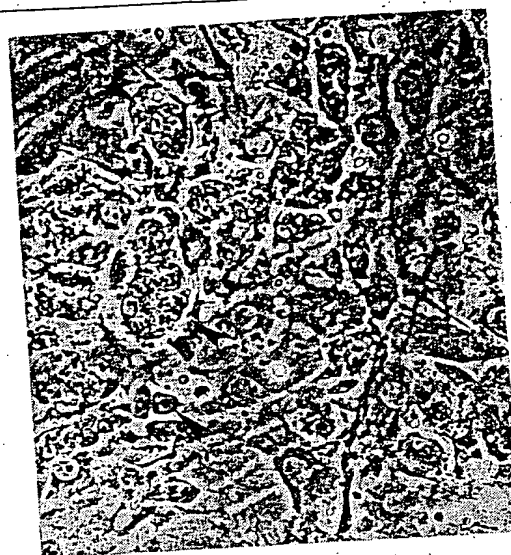


Fig. 1 Groups of pluripotent embryo cells (arrowed) growing in monolayer culture on a background of mitomycin C-inhibited STO cells. The isolation of a definite cell line from a blastocyst takes only ~3 weeks and the pluripotent cell colonies are visible within 5 days of passage. We have had 30% yield of lines from blastocysts in one experiment. Two of the lines have been rigorously cloned by single-cell isolation but most were only colony-picked—this makes no difference.

Previous attempts to obtain cultures of pluripotent cells directly from a mouse embryo have been unsuccessful<sup>3,4</sup>, although cells with a similar appearance have been reported to be present transiently<sup>5,6</sup>. We considered that success might depend on three critical factors: (1) the exact stage at which pluripotent cells capable of growth in tissue culture exist in the embryo; (2) explantation of a sufficiently large number of these precursor cells from each embryo; and (3) tissue culture in conditions most conducive to multiplication rather than differentiation of these embryonic cells. These considerations have been discussed at greater length elsewhere<sup>7</sup>. An indication of the optimal stage of embryonic development might be gained by a comparison of the properties of embryonic cells at various stages with established cultures of embryonal carcinoma (EC) cells. Cell-surface antigen expression and the patterns of protein synthesis revealed by two-dimensional electrophoresis have suggested that neither the cells of the 6½-day ectoderm nor those of the 3½-day inner cell mass show homology with EC cells, but that epiblast cells of the early post-implantation embryo at 5½ days post coitum may do so<sup>8</sup> (the day of finding coital plug is termed day ½). Cells from embryos of an early post-implantation stage seem to be the best candidates for direct progenitors of pluripotent cells in culture. As these embryos are difficult to isolate, and as the cell number in the isolated epiblast is small, we chose an alternative route to obtain embryo cells at this stage of development.

Mouse blastocysts may be induced to enter a state of diapause just before implantation. This delay in implantation depends on the maternal hormonal conditions, and may be induced experimentally by ovariectomy at an appropriate stage<sup>9</sup>. Embryos in implantational delay hatch from the zona but remain free-floating in the uterine lumen. A gradual increase in cell number occurs<sup>10</sup>, and the primary endoderm may be formed but no further development takes place until implantation occurs, under the control of hormonal stimuli.

129 SvE mice were caged in pairs and examined for mating plugs each morning. They were ovariectomized on the afternoon of day 2½ of pregnancy, injected subcutaneously with 1 mg. Depo-Provera (Upjohn), and delayed blastocysts were recovered 4-6 days later. The blastocysts were cultured intact in

groups of about six embryos in small drops of tissue culture medium under paraffin oil on tissue culture plastic Petri dishes for 4 days. The blastocysts attached within 48 h and the trophoblast cells grew out and differentiated into giant trophoblast cells. The inner cell mass cells subsequently developed into large egg cylinder-like structures, with a group of small round cells surrounded by endodermal cells growing attached to the Petri dish. The egg cylinder-like structures were picked off the dish, dispersed by trypsin treatment and passaged on to gelatin-pretreated Petri dishes containing mitomycin C-inactivated STO fibroblasts. All culture was carried out in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum and 10% newborn calf serum. The cultures were examined daily and passaged by trypsinization every 2–3 days. Actively proliferating colonies of cells closely resembling EC cells were apparent from an early stage. These colonies were picked out, passaged and mass cultures grown. The cell cultures had the appearance and general growth characteristics of feeder-dependent EC cells (Fig. 1).

The embryos used to initiate these cultures are from normal 129 SvE strain mice, that is, from the same strain of mice as many EC cell lines, in particular those grown in this laboratory. Therefore it was important to exclude any possibility of contamination of these cultures with EC cells from established cell lines. Cell cultures were established from different embryos in three separate experimental series, but the best indication of their separate identity came from their karyotype. Cultures were initiated from 6–12 embryos, thus it might be expected that both male and female cells should be present. None of the 129 embryonal carcinoma cell lines in this laboratory have a normal karyotype, and, in particular—in common with most available embryonal carcinoma cell lines—they do not contain a Y chromosome. These embryo-derived cells have a completely normal karyotype. An XY karyotype is shown in Fig. 2. Three additional cell lines have been analysed; two of these are normal 40XX and one is normal 40XY. We have termed these directly embryo-derived cells EK to distinguish them from EC cells. EK cells grow rapidly in culture and have been maintained for over 30 passages *in vitro*.

Cultures of EK cells were collected by trypsinization, and  $\sim 10^6$  cells injected subcutaneously into the flank of syngeneic male mice. Tumours grew in all cases, and histological examination of these revealed that they were teratocarcinomas. When the EK cells were passaged without feeder cells they formed embryoid bodies which, when kept in suspension, became cystic.



Fig. 2 Karyotype of an embryo-derived pluripotent cell line, 40XY. Over 80% of the spreads of this clonal line possessed 40 chromosomes and had a clearly identifiable Y chromosome.

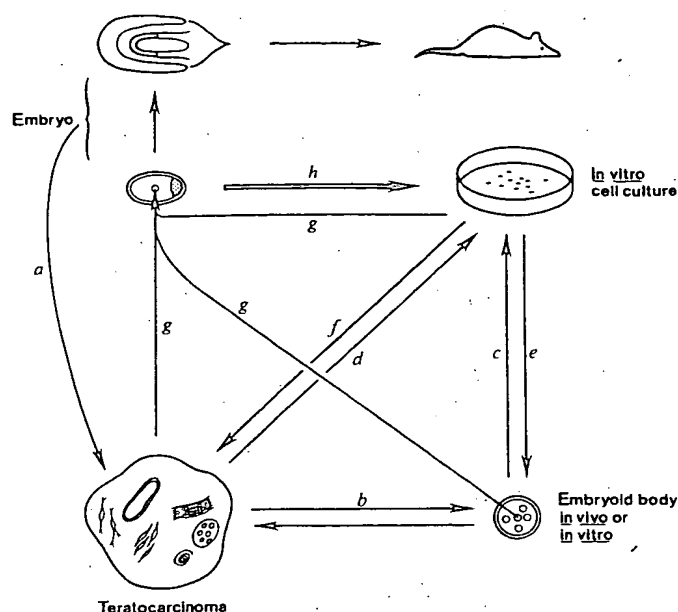


Fig. 3 Inter-relationships of cell lines, teratocarcinomas and embryoid bodies with normal mouse embryos. Arrows indicate routes of cell transfer: a, formation of teratocarcinoma by ectopic implantation of embryos; b, formation of embryoid bodies from teratocarcinoma and vice versa; c, derivation of cell culture from embryoid bodies; d, cell culture obtained directly from solid tumours; e, differentiation to embryoid bodies from culture; f, formation of solid tumours on reinjection of cells from culture; g, transfer of embryonal carcinoma cells either from cell culture or from the core of an embryoid body or from a solid tumour back to a blastocyst. All these procedures may result in chimaerism of the resulting mouse; h, the missing link supplied here.

Embryoid bodies allowed to attach to a Petri dish spread out and differentiated in the usual way into a complex of tissues. Preliminary observations indicate that, like early ectoderm cells of the mouse embryo and EC cells, EK cells carry the cell-surface antigens recognized by M1-22-25 (Forssman)<sup>8,11</sup> and anti-I Ma (lacto-N<sub>2</sub>-iso-octaosyl ceramide)<sup>12,13</sup> and also that two dimensional gel electrophoretic separations of their proteins very closely resemble those of the EC cell line PSMB.

We have demonstrated here that it is possible to isolate pluripotent cells directly from early embryos and that they behave in a manner equivalent to EC cells isolated from teratocarcinomas. The network of inter-relationships between the mouse embryo and pluripotent cells derived from it has previously lacked only the direct link between the embryo and cells in culture for completion. We have now demonstrated this (Fig. 3).

Teratocarcinoma cells are now being widely used as a model for the study of developmental processes of early embryonic cell commitment and differentiation. Their use as a vehicle for the transfer into the mouse genome of mutant alleles, either selected in cell culture or inserted into the cells via transformation with specific DNA fragments, has been presented as an attractive proposition. In many of these studies the use of pluripotent cells directly isolated from the embryos under study should have great advantages. We have now shown that these EK cell lines are readily established from cultures of single blastocysts and so far have 15 lines of independent embryonic origin, some of which have been isolated from non-129, outbred mouse stocks. We are now studying the chimaeric mice formed from these cells.

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1. Gardner, R. L. & Papaioannou, V. E. in *The Early Development of Mammals* (eds Balls, M. & Wild, A. E.) 107-132 (Cambridge University Press, 1975).
2. Stevens, L. C. *Dev Biol.* 21, 364-382 (1970).
3. Cole, R. J. & Paul, J. in *Prenatal Stages of Pregnancy* (eds Wolstenholme, G. E. W. & O'Connor, M.) 82-122 (Churchill, London, 1965).
4. Sherman, M. I. *Cell* 5, 343-349 (1975).
5. Atienza-Samols, S. B. & Sherman, M. I. *Dev Biol.* 66, 220-231 (1978).
6. Solter, D. & Knowles, B. *Proc. natn. Acad. Sci. U.S.A.* 72, 5099-5102 (1975).
7. Evans, M. J. *J. Reprod. Fert.* 62, 625-631 (1981).
8. Evans, M. J., Lovell-Badge, R. H., Stern, P. L. & Stinnakre, M.-G. *INSERM Symp.* 10, 115-129 (1979).
9. McLaren, A. *J. Endocr.* 50, 515-526 (1971).
10. Kaufman, M. H. in *Progress in Anatomy* Vol. 1 (eds Harrison, R. J. & Holmes, R. L.) 1-34 (Cambridge University Press, 1981).
11. Stern, P. L. *et al.* *Cell* 14, 775-783 (1978).
12. Feizi, T. *Blood Transfusion Immunohaemat.* 23, 563-577 (1980).
13. Kapadia, A., Feizi, T. & Evans, M. J. *Exp Cell Res.* 131, 185-195 (1980).

## Stage-specific embryonic antigen involves $\alpha 1 \rightarrow 3$ fucosylated type 2 blood group chains

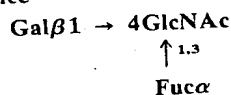
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There is much interest in developmentally regulated molecules which may have function in cell interactions and sorting during embryogenesis and differentiation. Numerous antisera have been raised which detect antigens that are expressed in early embryonic cells and become restricted during differentiation, being expressed in only a minority of adult cells (reviewed in refs 1-3). The precise antigenic determinants recognized by such antisera have not been defined. However, studies using a hybridoma antibody against mouse spleen cells<sup>4</sup> and monoclonal autoantibodies of patients with cold agglutinin disease<sup>5</sup> have shown that two defined carbohydrate antigen systems, the Forssman and the Ii antigens, have stage-specific expression in early mouse embryos. We now describe evidence that the stage-specific embryonic antigen SSEA-1 (ref. 6) involves the carbohydrate sequence



This determinant is formed by  $\alpha 1 \rightarrow 3$  fucosylation of blood group I or i antigens which are branched or linear oligosaccharides, respectively<sup>7-9</sup>, built of  $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}$  units and known as type 2 precursor chains<sup>10</sup> of the major blood group antigens. Thus, we introduce the concept of simple glycosylation changes as a basis for stage-specific expression of embryonic antigens.

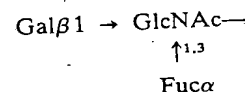
A relationship between the stage-specific embryonic antigen, SSEA-1, and blood group I antigen was suggested by: (1) some similarities in the distribution of these two antigens in early post-implantation embryos and teratocarcinomas<sup>5,11</sup>, and (2) the reactivities of anti-I and -SSEA-1 reagents with a glycosphingolipid fraction of erythrocytes<sup>12</sup>. Subsequent immunocytochemical studies (to be described elsewhere) have clearly shown that the two antigens are not identical. Furthermore, in binding assays using radiolabelled blood group I-active glycoproteins<sup>13</sup> isolated from sheep gastric mucins and human meconium, the anti-SSEA-1 reagent gave a binding curve only with the latter glycoprotein (Fig. 1).

The specificity of anti-SSEA-1 reagent was further investigated by inhibition assays using blood group substances with known A,B,H,Lewis<sup>x</sup>, Lewis<sup>y</sup>, I and i activities, some of which

are shown in Fig. 1. These substances were clearly divisible into two groups: those with potent inhibitory activities, giving 50% inhibition at  $0.3-6 \mu\text{g ml}^{-1}$ , and those with negligible or weak activities requiring concentrations  $>100 \mu\text{g ml}^{-1}$  for 50% inhibition. Almost all the substances with strong SSEA-1 activity lacked blood group ABH activities but expressed Le<sup>a</sup> and/or Ii activities.

The two ovarian cyst glycoproteins with the strongest SSEA-1 activity, designated N-1 10% and F1, contained 8.5% and 1.6% fucose, respectively<sup>14,15</sup>. In experiments to be described elsewhere, we showed that their reaction with anti-SSEA-1 was abolished by mild acid hydrolysis. Thus, it seemed likely that the SSEA-1 determinant involved fucose residue(s) and that the fucosyl linkage was other than those associated with A, B, H, Le<sup>a</sup> or Le<sup>b</sup> activities<sup>10</sup>. This agrees with previous evidence for the presence of unusual fucosyl glycopeptides<sup>20</sup> in undifferentiated cells of mouse embryos and teratocarcinomas.

Inhibition of binding assays were next performed with chemically synthesized<sup>21-23</sup> and natural oligosaccharides<sup>14,24</sup> containing type 1 ( $\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}$ ) or type 2 ( $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}/\text{Glc}$ ) precursor chain<sup>10</sup> sequences (Fig. 2). Whereas Ii-active synthetic tri- and pentasaccharides consisting of type 2 precursor chains were inactive as inhibitors of anti-SSEA-1, several fucose-containing oligosaccharides showed inhibitory activities. The most active inhibitor ( $\text{ID}_{50}$  0.5 nmol) was the oligosaccharide designated N-1 R<sub>L</sub> 0.71a, containing a fucosylated type 2 sequence:



This oligosaccharide was one of several isolated from glycoprotein N-1 after partial alkaline degradation<sup>14</sup>. The very poor inhibitory activity of 3-fucosyl lactose indicated that subterminal N-acetyl glucosamine is an important component of

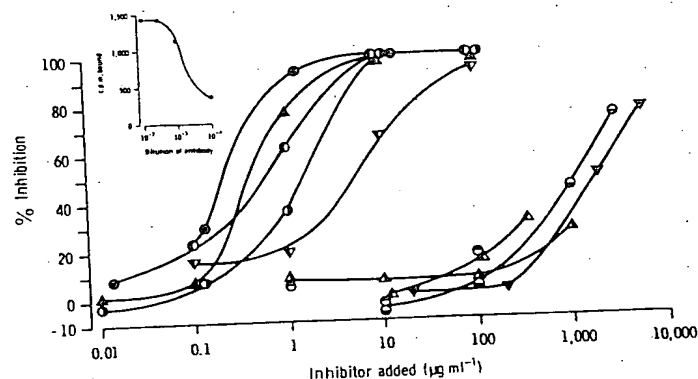


Fig. 1 Double antibody radioimmunoassay using anti-SSEA-1 and <sup>125</sup>I-labelled human meconium. The immunoassay procedure was a modification of that described previously<sup>13</sup>. The anti-SSEA-1 reagent<sup>6,11</sup> was ascites fluid from a pristane-primed BALB/c mouse injected with antibody-producing hybrid cells. Normal mouse serum, 1:100 dilution, was used as carrier and undiluted rabbit anti-mouse immunoglobulin serum (Dako-immunoglobulins) as second antibody. The binding curve is shown in the inset. Inhibition assays were performed using the anti-SSEA-1 reagent at 1:3,000 dilution. As inhibitors blood group substances of human and animal origins were used. Symbols, blood group activities and designations (the latter in parentheses) of representative samples are given below. The following were human ovarian cyst glycoproteins: ●, Le<sup>a</sup>I (N-1 10%)<sup>14</sup>; ▲, Ii (F1)<sup>15</sup>; ○, Ii (484)<sup>13</sup>; ▼, Le<sup>a</sup> (445); △, HLe<sup>b</sup> (JS)<sup>16</sup>; ⊙, A (438)<sup>13</sup>. Also tested were gastric mucosal glycoproteins from sheep, △, Ii (sheep 1 + 10)<sup>17</sup> and from hog, ▼, AH (hog A + H)<sup>18</sup>, a glycoprotein-rich extract from human meconium of non-secretor type, ○, Le<sup>a</sup>Ii (Mec)<sup>13</sup> and polyglycosylated ceramides isolated from human erythrocytes, ⊙, Hli (ref. 19).

## RAPID COMMUNICATION

# Pluripotent Embryonic Stem Cells from the Rat Are Capable of Producing Chimeras

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Embryonic stem cells have been enormously important in the production of targeted mutations in mice used in the study of gene function and biological aspects of disease states. The use of these cells for mouse studies is now widespread but the production of animals from similar cell lines derived from other species has not been previously reported. We demonstrate here the derivation of diploid rat embryonic stem cells (RESC-01). RESC-01 cells are SSEA-1 and alkaline phosphatase positive, grow best on primary rat embryonic fibroblasts, and can differentiate extensively *in vivo*. RESC-01 cells form cystic embryoid bodies capable of rhythmic contractions. Rat blastocysts injected with RESC-01 cells form chimeras. The results indicate that the successful *in vitro* propagation and chimera production with embryonic stem cells is not limited to the mouse. The long-term culture of rat ES cells will provide an important resource for the study of normal physiology and disease models where rat is the species of choice. © 1994 Academic Press, Inc.

The production of animals wholly derived from cultured embryonic stem (ES) cells by breeding chimeras revealed the enormous potential of ES cells for genetic analysis by combining their ability to colonize the germ line with methods for gene transfer (Bradley *et al.*, 1984), insertional mutagenesis (Zhou *et al.*, 1993), and, most recently, for targeted disruption of specific genes (Koller and Smithies, 1992). ES-like cells have been isolated from other species; however, only ES cell lines from the mouse have been brought into routine usage for genetic modification and germ line chimera production. Thus, the creation of animal models with homologous recombination using mammalian species other than the mouse is not currently possible.

Recently, our lab has developed methods for culturing preimplantation rat embryos. This has required the establishment of complex culture media and optimal cul-

ture conditions (Ng and Iannaccone, 1992; Van Winkle *et al.*, 1990). The combination of these techniques with the development of methods to reliably establish and date pseudopregnancy in the rat has allowed us to produce aggregation chimeras between two different embryos with high efficiency. By extending these techniques and combining them with procedures in use to derive and maintain mouse ES cells, we have now established the continuous culture of cells from the rat blastocyst. These rat cells, unless cultured on rat primary embryonic fibroblast feeder layers or in the presence of leukemia inhibitor factor (LIF), will differentiate extensively. Like mouse ES cells the RESC-01 (diploid rat embryonic stem cells) cells form embryoid bodies if grown in suspension and form teratomas with extensive differentiation when injected into nude mice. Most importantly, we have demonstrated that the rat cells can be used to produce chimeras by injection into rat blastocysts. Based on these criteria, we believe that these cells are the rat equivalent of mouse pluripotent embryonic stem cells. The derivation of ES cells from the rat could provide a route for the production of models for human disease, such as hypertension and heart disease, where the rat is the species of choice.

A total of 420 blastocysts from the inbred PVG strain of black hooded rats were placed on rat embryonic fibroblast feeder layers in organ culture dishes using rat embryo medium (see legend to Fig. 1). Of these, 192 blastocysts attached and after 3 days all of the attached blastocysts had expanded inner cell mass populations. These were mechanically disrupted and individually placed on new feeder layers. Mechanical disruption was repeated daily and colonies of ES-like cells were identified after 5 days. Forty-eight individual cell cultures were created in this way. We expanded the number of cells from one of these chosen at random to create the cell line RESC-01 which has a modal chromosome number of 42, XY (Fig. 1).

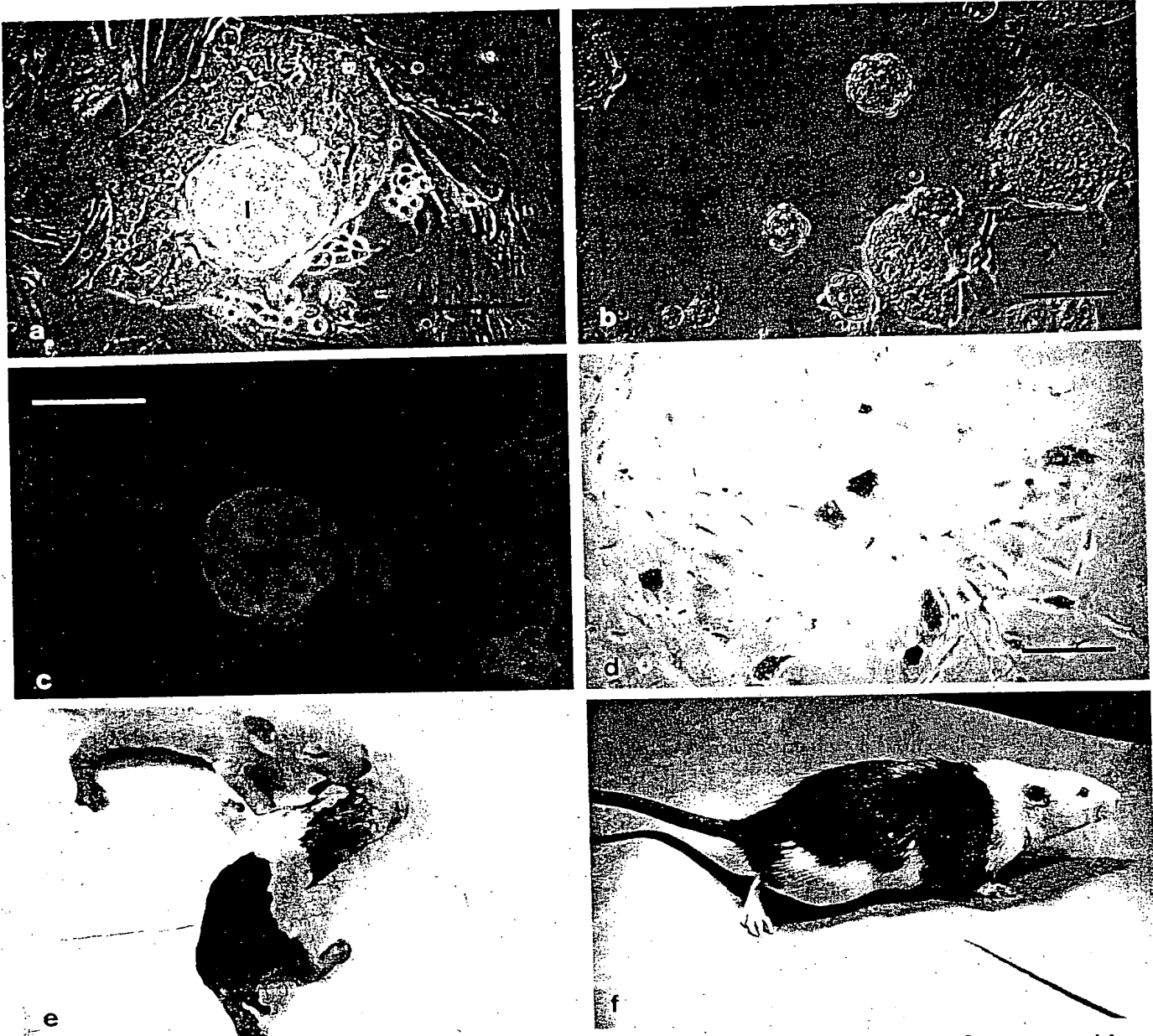


FIG. 1. (a) Phase-contrast photomicrograph of attached rat blastocysts. Rat blastocysts (PVG-RT1<sup>c</sup> × PVG-RT1<sup>c</sup>) were removed from the uterus by flushing with a balanced salt solution (Ng and Iannaccone, 1992). The blastocysts were placed on primary rat embryonic fibroblasts in organ culture dishes containing Markert's modification of Whittingham's medium (Yamamura and Markert, 1981) supplemented with 20% FBS (Intergen, lot screened for mouse ES cell growth), penicillin (100 units/ml)/streptomycin (100 µg/ml), 1% nonessential amino acids (NEAA, 100× stock from GIBCO, 320-1140PG), 1% nucleoside from stock (100× stock = adenosine 3.0 M, guanosine 3.0 M, cytidine 2.3 M, uridine 3.0 M, thymidine 1.0 M), 1% 2-β-mercaptoethanol stock (stock = 7 µl/10 ml PBS), 2 mM L-glutamine, and LIF (2000 units/ml Esagro, GIBCO). T, trophectoderm; I, inner cell mass; F, HREF feeder layer; bar, 100 µm. (b) Phase-contrast photomicrograph colonies of RESC-01 cells following trypsinization at passage 7 grown in DMEM supplemented as in (a); bar, 100 µm. (c) Epifluorescent photomicrograph of RESC-01 cells (passage 7) following incubation with antibodies against SSEA-1; HREF feeder layers do not react with the antibody; irrelevant first antibody controls were negative; positive controls included D3 mouse ES cells; bar, 40 µm. (d) Phase-contrast photomicrograph of RESC-01 colonies (passage 7) on HREF feeder layers and stained for alkaline phosphatase. The RESC-01 cells, but not the HREF feeder layer cells, are alkaline phosphatase positive. Similar results are obtained at passage 12; bar, 200 µm. (e) Chimera 4999 (female) photographed when 9 days old. (f) Chimera 5001 (male) photographed when 100 days old.

The growth and differentiation of RESC-01 cells were analyzed using various substrata and media. RESC-01 cells were plated at low density and grown on plastic, gelatin-coated plastic, growth-arrested STO mouse fibroblast feeder layers, and growth-arrested HREF (Holtzman strain rat embryonic fibroblast) fibroblast feeder layers. Differences in growth curves on HREF, STO, gelatin-coated plastic, and plastic were statistically significant (data not shown). Cell counts show growth was best on HREF feeder layers. In separate experiments cells were plated on several substrata or in media containing various concentrations of LIF, which has been shown to inhibit the differentiation of mouse ES cells in culture even in the absence of embryonic fibroblast feeder layers (Mummery *et al.*, 1990). When RESC-01 cells were grown in the absence of LIF or on plastic the population rapidly differentiated into cells which were morphologically distinct from undifferentiated ES cells.

The ability to maintain these cells in the undifferentiated state was compared on various substrata (Fig. 2). There was a statistically significant difference in the number of colonies which contained differentiated cells on the various substrata. On Day 2 and Day 3 following plating the least differentiation occurred on HREF, followed by STO fibroblasts and gelatin-coated plastic. Plating on plastic led to the most rapid differentiation of colonies. Growth on HREF fibroblasts or gelatin-coated plastic in the presence of various concentrations of mouse LIF demonstrated a concentration-dependent inhibition of differentiation of RESC-01 cells (Fig. 2) without affecting the rate of growth. The number of colonies in which any of the cells had differentiated was determined. The cells were plated as small clumps of 2-4 cells at low density (50,000 cells/plate) so that 50 individual colonies could be scored on the basis of morphology. Differentiation appears to be related to colony size (data not shown) and by plating single cells the undifferentiated state can be maintained for extended periods.

The spontaneous differentiation of mouse ES cells in suspension results in the formation of cystic structures which eventually develop several cell layers reminiscent of the early embryo, known as embryoid bodies. We established embryoid bodies in this manner from passage 6 RESC-01 cells. After 7 days some of the rat cystic embryoid bodies acquired complex shapes with cystic fluid filled cavities and multiple cell layers, which on histological section appeared endoderm-like and ectoderm-like. Some of these began rhythmic contractions like those produced with mouse embryoid bodies (Sanchez *et al.*, 1991). RESC-01 cells at passage 10, 11, or 12 injected into nude mice also resulted in extensive differ-

entiation of the cells. Teratomas formed within 3 weeks which contained differentiated cells and tissues derived from all three embryonic germ layers. These included muscle, neural tissues, bone, cartilage, bone marrow, respiratory and gastrointestinal mucosa, squamous epithelium, and pancreatic acini (data not shown).

Cells isolated from cultures of undifferentiated RESC-01 cells (passage 5 or 6) were microinjected into blastocysts isolated from Day 4 pregnant Holtzman strain rats. From 10 to 30 RESC-01 cells were injected into each blastocyst and the injected blastocysts were transferred into the uteri of Holtzman albino strain female rats in their third day of pseudopregnancy (Weinberg *et al.*, 1985; Ng and Iannaccone, 1992). These procedures resulted in a pregnancy rate of 79% and a live birth rate of 39%. Eighty-nine albino pups were born and six of these displayed a patchy mixture of albino, black, and agouti coat colors (Fig. 1). These six vary from approximately 10 to 80% pigmented coat. Two are male and four are female. They all display posterior pigmentation, never seen in the PVG (hooded) or Holtzman rats. Patches of agouti and black are apparent in pigmented areas of the chimeras. Two have pigmented patches on the head, while PVG coat pattern always consists of pigmentation on the entire head. One has bilateral pigmentation on the dorsal surface of the distal forelimbs. These coat color patterns could only have occurred as a result of chimera formation between the injected RESC-01 cells and the Holtzman strain blastocyst. The patterns observed in our rat ES cell chimeras are consistent with those previously reported in aggregation chimeras formed by amalgamation of 8-cell embryos from strains genetically similar to these used here (Yamamura and Markert, 1981).

Pluripotent cells have been isolated from mink (Sukoyan *et al.*, 1992), pig (Notarianni *et al.*, 1991), and hamster (Doetschman *et al.*, 1988) but so far there are no published accounts of chimera formation with stem cells from species other than mouse. The ability to establish a stem cell population from the rat and make chimeras with them is the first step toward providing an important addition to the repertoire of genetic manipulation techniques in mammals. The creation of targeted mutations in the mouse has been a valuable source of animal models of human disease. For many diseases, however, the physiology of the mouse is either poorly understood or inappropriate for experiments germane to the particular disease. For example, the study of cardiovascular disease has extensively utilized the rat because of its small size and its appropriate physiological responses to experimental manipulations. For similar reasons the rat is important in the study of lipid transport, atherosclerosis, hypertension, and cardiomyopa-



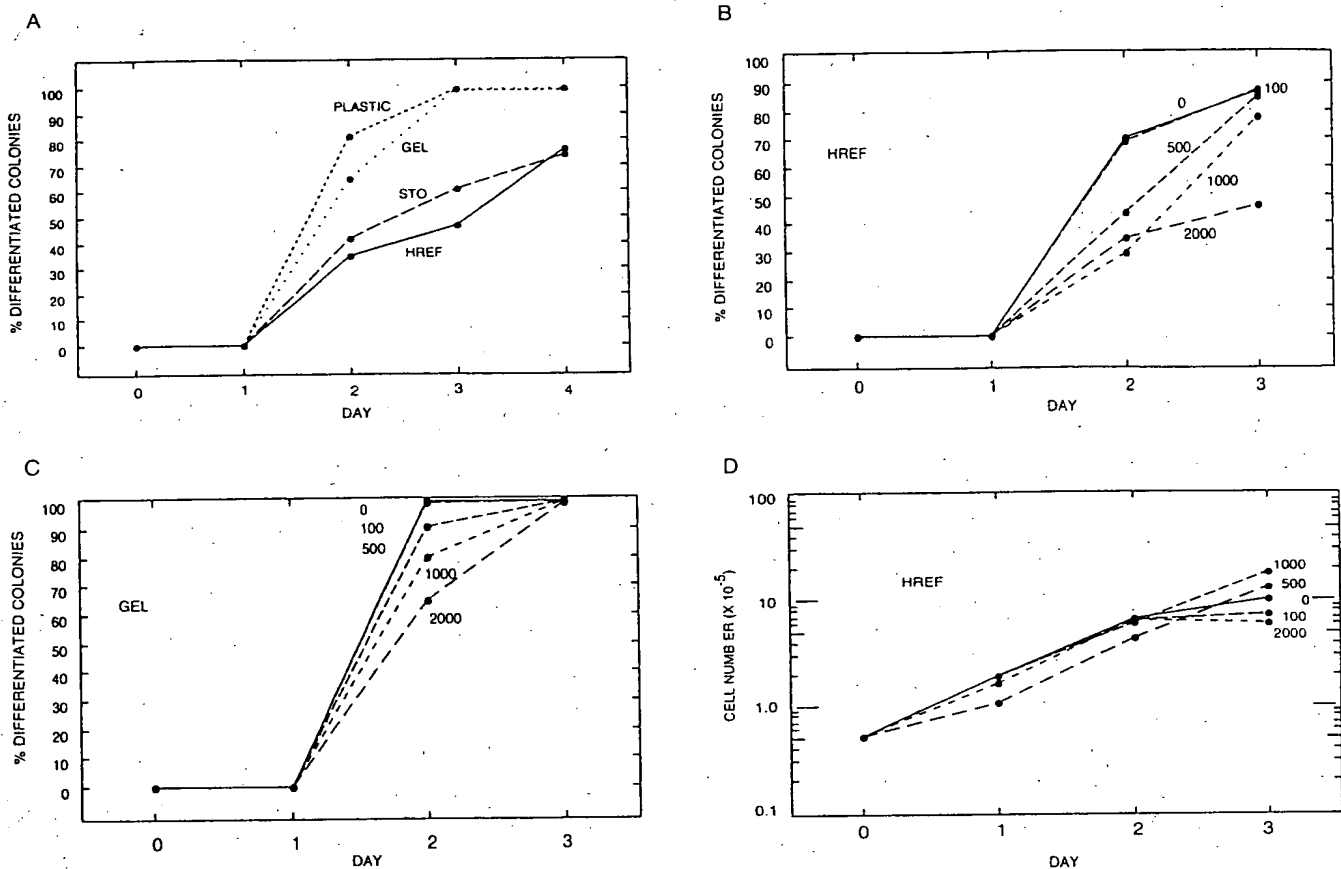


FIG. 2. (A) Differentiation of colonies of RESC-01 cells on various substrata. Standard error of the means falls within the range of the symbols. The proportion of differentiated colonies of RESC-01 cells grown on HREF is significantly different from those grown on STO on Day 2 ( $P = 0.02$ ); from those grown on gelatin-coated plastic on Days 2, 3, and 4 ( $P < 0.001$ ); and from those grown on plastic on Days 2, 3, and 4 ( $P < 0.001$ ). The proportion of differentiated colonies of RESC-01 cells grown on STO is significantly different from those grown on gelatin-coated plastic on Days 2, 3, and 4 ( $P < 0.001$ ) and from those grown on plastic on Days 2, 3, and 4 ( $P < 0.001$ ). The proportion of differentiated colonies of RESC-01 cells grown on gelatin-coated plastic is significantly different from those grown on plastic on Day 2 ( $P < 0.003$ ). (B and C) Differentiation of RESC-01 colonies on HREF fibroblasts or gelatin-coated plastic. Fifty colonies were counted in six determinations. The numbers by each curve represent the concentration of mouse LIF in units/ml. The proportion of differentiated colonies of RESC-01 cells grown of HREF in the absence of LIF is significantly different in the presence of 500 units/ml LIF on Days 2 and 3 ( $P < 0.001$ ); in the presence of 1000 units/ml LIF on Day 2 ( $P < 0.001$ ); and in the presence of 2000 units/ml LIF on Days 2 and 3 ( $P < 0.006$ ). The proportion of differentiated colonies of RESC-01 cells grown on gelatin-coated plastic in the absence of LIF from those grown in the presence of 500 units/ml of LIF on Day 2 ( $P < 0.0001$ ); in the presence of 1000 units/ml of LIF on Day 2 ( $P = 0.007$ ); and in the presence of 2000 units/ml of LIF on Day 2 ( $P = 0.0001$ ). Cells were prepared by trypsinization and plated on the indicated substrata at passage 6. The curves were produced by plating 50,000 cells as clumps of 2-3 cells each on  $10^6$  growth-arrested embryonic fibroblasts. Fifty colonies were scored in six determinations made on duplicate plates. Any differentiated cells (epithelial, mesenchymal, or endodermal morphologies) in a colony caused the colony to be scored as differentiated. (D) Growth curves in the presence of various concentrations of LIF. LIF did not influence proliferation of these cells.

thy. Detailed information from behavioral studies are much more readily available in the rat than in the mouse. Therefore, the availability of ES cells from the rat, by extending the scope of studies to include targeted mutational analysis, will be critical to furthering our understanding of a variety of disease states. Nevertheless, the probability of obtaining an undifferentiated continuously proliferating cell line is certainly less than

in the mouse. Strain differences in the frequency of establishing continuous ES cell lines in the mouse are well known, and although it is reasonable to suppose that this is also true in the rat, at this time we have no formal evidence that it is. These are important considerations since as yet our chimeras have not demonstrated germ line transmission. While several of the animals reported here have shown limited reproductive capacity, it is



likely that additional cell lines will have to be established in order to fully utilize ES cell technologies in the rat. By extending the studies reported here and by continuing to work with species other than the mouse it will be possible to resolve many of these issues and bring the valuable genetic resources of ES cell technology to other mammalian species.

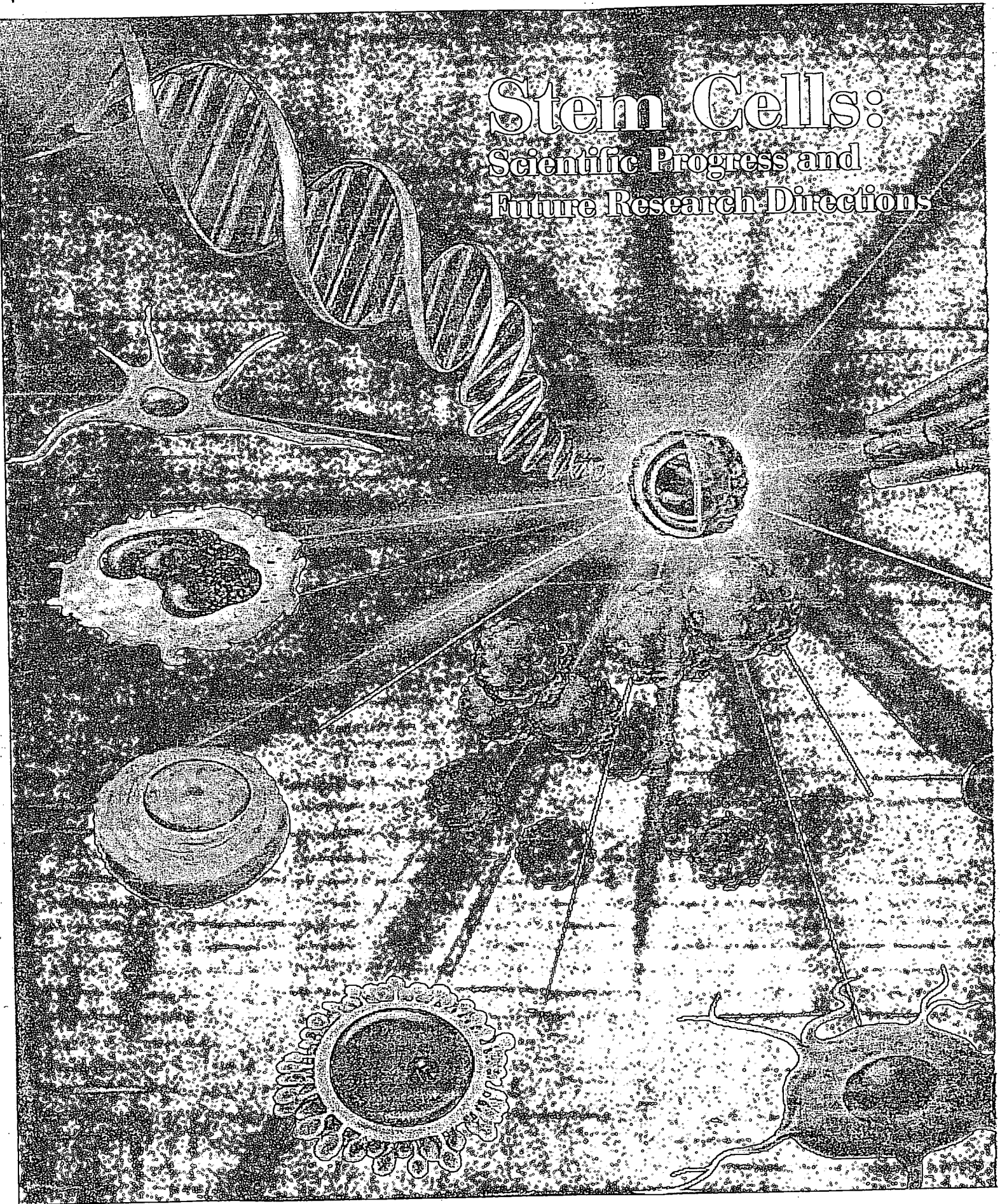
The authors express their appreciation to Michael Kuehn for his constant support, encouragement and advice throughout these studies and for his critical review of this paper, to Karen Mason for the karyotypes, and to Jeanne Loring for the SSEA-1 and alkaline phosphatase protocols developed for similar cells. We are grateful for financial support from the Lucille Markey Charitable Trust and the Feinberg Cardiovascular Institute of Northwestern University Medical School, Francis Klocke, Director.

#### REFERENCES

- Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* 309, 255-256.
- Doetschman, T., Williams, P., and Maeda, N. (1988). Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev. Biol.* 127, 224-227.
- Koller, B. H., and Smithies, O. (1992). Altering genes in animals by gene targeting. *Annu. Rev. Immunol.* 10, 705-730.
- Mummary, C. L., Feyen, A., Freund, E., and Shen, S. (1990). Characteristics of embryonic stem cell differentiation: A comparison with two embryonal carcinomas cell lines. *Cell Differ. Dev.* 30, 195-206.
- Ng, Y. K., and Iannaccone, P. M. (1992). Experimental chimeras: Current concepts and controversies in normal development and pathogenesis. In "Current Topics in Development Biology" (R. A. Pederson, Ed.), pp. 235-274. Academic Press, San Diego.
- Notarianni, E., Galli, C., Laurie, S., Moor, R. M., and Evans, M. J. (1991). Derivation of pluripotent, embryonic cell lines from the pig and sheep. *J. Reprod. Fertil. Suppl.* 43, 255-260.
- Sanchez, A., Jones, W. K., Gulick, J., Doetschman, T., and Robbins, J. (1991). Myosin heavy chain gene expression in mouse embryoid bodies. *J. Biol. Chem.* 266, 22419-22426.
- Sukoyan, M. A., Golubitsa, A. N., Zhelezova, A. L., Shilov, A. G., Vatoлин, S. Y., Maximovsky, L. P., Andreeva, L. E., McWhir, J., Pack, S. D., Bayborodin, S. I. et al. (1992). Isolation and cultivation of blastocyst-derived stem cell lines from American mink (*Mustela vison*). *Mol. Reprod. Dev.* 33, 418-431.
- Van Winkle, L. J., Iannaccone, P. M., Campione, A. L., and Garton, R. L. (1990). Transport of cationic and zwitterionic amino acids in preimplantation rat conceptuses. *Dev. Biol.* 142, 184-193.
- Weinberg, W. C., Howard, J. C., and Iannaccone, P. M. (1985). Histological demonstration of mosaicism in a series of chimeric rats produced between congenic strains. *Science* 227, 524-527.
- Yamamura, K-I., and Markert, C. L. (1981). The production of chimeric rats and their use in the analysis of the hooded pigmentation pattern. *Dev. Genet.* 2, 131-146.
- Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. M., and Kuehn, M. (1993). Nodal is a novel TGF- $\beta$ -like gene expressed in the mouse node during gastrulation. *Nature* 361, 543-547.

# Stem Cells:

Scientific Progress and  
Future Research Directions



**REPORT PREPARED BY THE NATIONAL INSTITUTES OF HEALTH**

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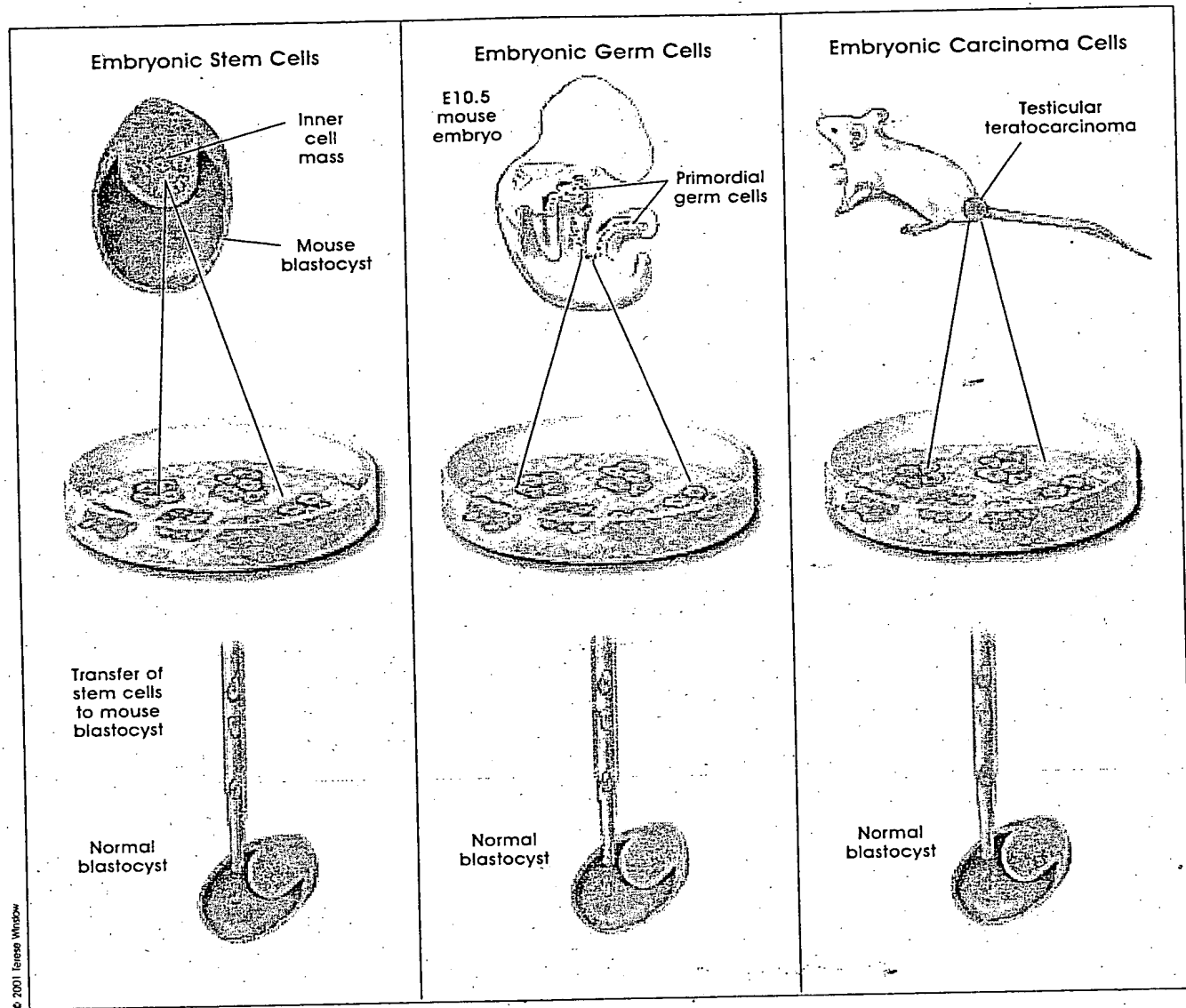


Figure B.1. Origins of Mouse Pluripotent Stem Cells.

through hundreds of population doublings, and still maintain a normal complement of chromosomes, called a karyotype [31, 35].

## MAINTAINING MOUSE EMBRYONIC STEM CELLS IN THEIR UNDIFFERENTIATED STATE

### Leukemia Inhibitory Factor and STAT3 Activation

Mouse ES cells can be maintained in a proliferative, undifferentiated state *in vitro* by growing them on

feeder layers of MEF cells. An alternative to culture on feeder layers is the addition of leukemia inhibitory factor (LIF) to the growth medium [31, 39]. LIF is produced by feeder cells and, in their absence, allows mouse ES cells *in vitro* to continue proliferating without differentiating [20]. LIF exerts its effects by binding to a two-part receptor complex that consists of the LIF receptor and the gp130 receptor. The binding of LIF triggers the activation of the latent transcription factor STAT3, a necessary event *in vitro* for the continued proliferation of mouse ES cells [5, 12, 14]. Recent



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## Product Information

**Leukemia Inhibitory Factor (LIF)**  
**Human, Recombinant**  
 Expressed in *E. coli*

Product No. L5283

### Description

Leukemia Inhibitory Factor (LIF), a 20 kD protein containing 180 amino acid residues, is a multifunctional glycoprotein that induces macrophage differentiation and suppresses the proliferation of the murine M1 myeloid cell line.<sup>1</sup> Synonyms for LIF include: differentiation-inhibitory activity (DIA) for murine embryonic stem (ES) cells,<sup>2,3</sup> human Interleukin for DA cells (HILDA),<sup>4</sup> hepatocyte stimulating factor III,<sup>5</sup> cholinergic neuronal differentiation factor,<sup>6</sup> and lipoprotein lipase inhibitor.<sup>7</sup> Leukemia inhibitory factor plays an important role, along with interleukin-6 (IL-6) and granulocyte-colony stimulating factor (G-CSF), in the regulation of early hematopoietic stem cells.<sup>8</sup> It is also important in the release of calcium from bone tissue.<sup>9</sup>

### Performance Characteristics

The biological activity of recombinant, mouse LIF is measured by its ability to stimulate the differentiation of the M1 mouse myeloid leukemic cell line. The specific activity is not less than  $1 \times 10^8$  units/mg, where 50 units is defined as the amount of mouse LIF required to induce differentiation in 50% of the M1 colonies in 1 ml agar cultures.

### Product Information

Expressed in *E. coli*  
 Molecular Weight: 20 kD  
 Purity:  $\geq 95\%$  by SDS-PAGE and HPLC  
 Specific Activity:  $\geq 1 \times 10^8$  units/mg  
 Package Size: 10  $\mu$ g/vial  
 Volume/vial: 1 ml

Formulation: 0.2  $\mu$ m-filtered phosphate buffered saline containing 0.02% Tween 20.  
 Carrier Protein: none  
 Sterility: 0.2  $\mu$ m-filtered, aseptic fill  
 Endotoxin:  $\leq 0.1$  ng/ $\mu$ g LIF

### Dilution and Use

Dilute the contents of the vial using 0.2  $\mu$ m-filtered PBS containing 1% BSA to a final concentration of not less than 1.0  $\mu$ g/ml.

### Storage

Store at 2-8°C.

### References

1. Gearing, D., et al., EMBO J., 6, 3995 (1987).
2. Smith, A., et al., Dev. Biol., 121, 1 (1987).
3. Smith, A., et al., Nature, 336, 688 (1988).
4. Moreau, J., et al., Nature, 336, 690 (1988).
5. Baumann, H., et al., J. Immunol., 143, 1163 (1989).
6. Yamamori, T., et al., Science, 246, 1412 (1989).
7. Mori, M., et al., Biochem. Biophys. Res. Commun., 160, 1085 (1989).
8. Leary, A.G., et al., Blood, 75, 1960 (1990).
9. Abe, E., et al., Proc. Natl. Acad. Sci., USA, 83, 5958 (1986).
10. Kitamura, T., et al., J. Cell Physiol., 140, 323 (1989).
11. Kuwaki, T., et al., Biochem. Biophys. Res. Commun., 161, 16 (1989).

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